AOPPs Induce MCP-1 Expression by Increasing ROS-Mediated Activation of the NF-κB Pathway in Rat Mesangial Cells: Inhibition by Sesquiterpene Lactones

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Key Words
MCP-1 • Advanced oxidation protein products • Reactive oxygen species • Nuclear factor-kappa B • Sesquiterpene lactones

Abstract
Background: Monocyte chemoattractant protein-1 (MCP-1) plays an important role in extracellular matrix accumulation through macrophage recruitment and activation in the development and progression of diabetic nephropathy. Therefore, this study examined whether advanced oxidation protein products (AOPPs) are involved in nuclear factor-κB (NF-κB) activation and MCP-1 mRNA and protein expression in mesangial cells (MCs) and evaluated the effects of derivatives of sesquiterpene lactones (SLs) on AOPP-induced renal damage.

Methods: MCP-1 mRNA and protein expression in MCs were determined by quantitative real-time PCR and ELISA, respectively. The level of intracellular reactive oxygen species (ROS) was determined by flow cytometry. The protein expression of tubulin, P47, NF-κB p65, phospho-NF-κB p65, IκB, phospho-IκB, IκKB and phospho-IκKB was evaluated by Western blot.

Results: AOPPs caused oxidative stress in MCs and activated the NF-κB pathway by inducing IκBα phosphorylation and degradation. Inhibition of ROS by SOD (ROS inhibitor) blocked the AOPP-mediated NF-κB pathway. Moreover, the inhibition of AOPP-induced overproduction of MCP-1 mRNA and protein was associated with inhibition of IκBα degradation by SLs.

Conclusion: AOPPs induce MCP-1 expression by activating the ROS/NF-κB pathway and can be inhibited by SLs. These findings may provide a novel approach to treat inflammatory and immune renal diseases, including diabetic nephropathy.
Introduction

Diabetic nephropathy (DN) is one of the most prevalent and serious microvascular complications of diabetes and is a leading cause of end-stage renal disease (ESRD) [1]. The pathologic features of DN include mesangial and matrix glomerular hypertrophy and thickening of the glomerular basement membrane (GBM), which results in progressive renal impairment and glomerulosclerosis [2]. Glomerular mesangial cells are considered to be important players in the pathogenesis of DN [3] because mesangium expansion is consistently observed in DN patients, and the ever-increasing extracellular matrix (ECM) deposition has generally been considered to be critical in the pathogenesis of the disease [2]. Previous studies have indicated that MCs overexpress ECM in the presence of a high concentration of glucose and advanced glycation end products [3, 4]. Monocyte chemotactic protein-1 (MCP-1), which is a member of the C-C chemokines, is mainly secreted by MCs and upregulated in many renal diseases, including DN [5, 6]. MCP-1 is involved in the pathogenesis of DN, particularly inflammation, which is regulated by NF-κB signaling [7, 8]. The role of MCP-1 in ECM accumulation under diabetic conditions suggests that this protein may be involved in ECM synthesis [5].

Advanced oxidation protein products (AOPPs) are a novel class of renal pathogenic mediators that have been found to be involved in not only obesity and metabolic syndrome but also diabetic patients with or without microvascular complications [9]. AOPPs are uremic toxins that were first identified and reported by Witko-Sarsat in 1996 and contain dityrosine and cross-linking protein products formed during oxidative stress as a result of a reaction between chlorinated oxidants and plasma proteins. They are considered to be the true mediators of the proinflammatory effects of oxidative stress in uremia [10, 11]. Taken together, these data suggest that oxidized proteins can induce oxidative stress. However, whether AOPP accumulation affects mesangial cell function and its main signaling pathway is not clear. Thus, in the present study, we examined whether AOPP-induced reactive oxygen species (ROS) and the main signaling pathway are involved in NF-κB activation as an approach for understanding the upstream regulation of AOPP-induced NF-κB activation in MCs. We also evaluated the role of NF-κB activation in MCP-1 mRNA and protein expression in MCs cultured in the presence of AOPPs to determine the biologic significance of NF-κB activation.

Parthenolide, which is a natural sesquiterpene lactone (SL) isolated from the medicinal herb feverfew, is used to treat migraines, inflammation and arthritis [12]. Parthenolide and other SLs have been reported to have anti-inflammatory activities by inhibiting NF-κB, and parthenolide has been reported to inhibit nuclear factor kappa-B kinase (IKK) and/or directly inactivate NF-κB [13, 14]. Other studies have indicated that parthenolide blocks MCP-1 mRNA and protein expression, which are associated with inhibition of IKK activity, preservation of IκB and inhibition of NF-κB translocation [7]. Recent studies have demonstrated that PTL can inhibit inflammatory factors in human renal mesangial cells under hyperglycemic conditions [15]. Jordan et al. reported that PTL can selectively eradicate acute myeloid leukemia (AML) stem and progenitor cells through NF-κB inhibition and ROS generation [16], and the water-soluble form of PTL (DMPAT) is currently being tested in a clinical trial. Micheliolide (MCL), which is a naturally occurring guaianolide sesquiterpene lactone, can also selectively inhibit AML stem and progenitor cells. Moreover, its water-soluble form, DMAMCL, has been demonstrated to have superior efficacy compared to DMPAT for treatment in an acute leukemia mice model [17].

In the present study, we investigated the molecular mechanisms underlying AOPP-induced MCP-1 expression in cultured rat MCs. Here, we show that the AOPP-induced expression of MCP-1 in MCs is mediated through activation of the NF-κB signaling pathway, following ROS generation. Furthermore, some SLs and their derivatives (Fig. 1A) were found to prevent NF-κB activation and subsequent MCP-1 mRNA and protein expression.
Materials and Methods

**AOPPs-RSA Preparation and Determination**

AOPPs-rat serum albumin (AOPPs-RSA) was prepared according to a previously described method. Briefly, RSA solution (100 mg/ml) was exposed to 200 mM HOCl at a molar ratio of 1/140 for 30 min at 37°C and was then dialyzed against phosphate-buffered saline (PBS) to remove free HOCl. The AOPP preparation was passed through a Detoxi-Gel column (Pierce, USA) to remove contaminated endotoxin. The endotoxin levels in the preparation were measured with a Limulus Amoebocyte Lysate kit (Sigma-Aldrich, USA) and were found to be below 0.025 EU/ml. The AOPP content was determined by measuring the absorbance at 340 nm in an acidic condition and was calibrated with chloramines-T in the presence of potassium iodide. The AOPP content in the AOPPs-RSA and unmodified RSA was 57.88±4.21 and 0.2±0.06 nmol/g protein, respectively.

**Cell Culture**

Rat glomerular MCs (HBZY-1, Life-Science Academy of Wuhan University, Wuhan, China) were cultured and maintained in DMEM, containing 5.6 mmol/L glucose supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO₂ at 37°C. The mesangial cells were serum-deprived for 24 h prior to the experiment. The cells were cultured in a 100-mm culture dish for western blot analysis on a cover glass coated with polylysine for intracellular ROS measurement and a 6-well culture plate for quantitative real-time PCR and ELISA.

**Determination of Intracellular ROS by Flow Cytometry**

The level of intracellular ROS was determined by measuring samples with the oxidation-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, USA). This compound readily diffuses into cells and is deacetylated to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH), which
emits fluorescence when DCFH reacts with ROS to form the highly fluorescent 2',7'-dichlorofluorescein (DCF). The cells were washed with D-Hank’s solution and then incubated with 10 μM of DCFH-DA for 30 min at 37°C. The distribution of DCF fluorescence in the cells was detected using a flow cytometer with a wavelength of 488 nm for excitation and 525 nm for emission.

**Measurements of MCP-1 by ELISA**

Culture supernatants were collected from various experimental conditions, centrifuged to remove the cell debris, and stored at -80°C for analysis. The MCP-1 protein concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Ebioscience Inc., USA). According to the manufacturer’s instructions, the ELISA kit for MCP-1 is specific for rat MCP-1 and has a sensitivity of 4.7 pg/ml. The standard curve range was 7.8-500 pg/ml, and the results were corrected for the cell count.

**Quantitative Real-time PCR (qPCR) Analysis**

To validate MCP-1 gene expression in all of the cell samples, the total RNA was isolated from the cell lines, and cDNA synthesis was performed using TRIzol and Oligo d(T) (Invitrogen, USA). The qPCR assay was performed using the SYBER Green assay (Applied Biosystems, USA). The amplification conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Thermal cycling and fluorescence detection were performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA). MCP-1 mRNA expression relative to GAPDH expression was determined using the ΔCt method. All of the primers for qRT-PCR were designed with Primer Express software (ABI). The primer sequences were as follows: MCP-1 (±) TAGCATCAGCATGGCTGTC/ CCGACTCATGGGATCATCT and GAPDH (±) ATTGTCAGGAAGATGCTGG/ ATGGACTGTGGTCATGAGC. The expression level was analyzed using the 2-DDCt method.

**Western blot analysis**

All of the samples were rinsed three times with ice-cold PBS and dissolved in lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF) for 25 min on ice. The lysates were centrifuged (12,000 x g) at 4°C for 15 min, and then the proteins were boiled in loading buffer for 5 min and blotted onto a polyvinylidene difluoride (PVDF) membrane after they were separated on a 10% Tris-glycine gradient gel. The non-specific binding sites were blocked by immersing the membrane in 5% non-fat milk in Tris-buffered saline (TBS) solution for 1 h and then incubated with the primary antibody overnight at 4°C. The membrane was then washed in Tris-buffered saline (TBS) with 0.01% Tween 20 before being incubated with the respective secondary antibody for 1 h at room temperature. Electrochemiluminescence (ECL, Amersham Biosciences, USA) detection was performed, and the images were captured and documented using a CCD system (Imagestation 2000MM, Kodak, USA). The quantitative analysis of these images was performed using ImageJ, and the optical density was normalized against tubulin.

The following primary antibodies were used: anti-NF-κB p65, anti-phospho-NF-κB p65, anti-IκB-α, anti-phospho-IκB-α, anti-IKK-β, anti-phospho-IKK-β (Cell Signaling Technology, USA), and anti-P47phox (Santa Cruz Biotechnology, USA).

**Data analysis**

The results are expressed as the mean ± standard error of the mean (SEM) from at least three independent experiments. The statistical significance of the differences between the test groups was assessed by one-way analysis of variance (ANOVA) followed by Scheffe’s test (post-hoc). Statistical significance was defined as P< 0.05.

**Results**

**AOPPs induce NADPH oxidase-dependent ROS production in MCs**

To determine whether AOPPs could induce ROS production in MCs, we used the fluorescent probe DCFH-DA to measure ROS generation in MCs. We found that treatment with AOPPs induced a significant increase in ROS (Fig. 2A). NADPH oxidase is an important source of ROS production. Therefore, to determine whether ROS was generated by NADPH oxidase,
the activated form of NADPH oxidase was assessed. The activated form of NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits (p40phox, p47phox and p67phox). Phosphorylation of p47phox leads to a conformational change, which allows for its interaction with p22phox and is indicative of NADPH activation [18]. Therefore, we determined the effect of AOPPs on NADPH oxidase activation by assessing the translocation of p47phox in MCs (Fig. 2B). Our results showed that AOPPs induce a significant p47phox membrane translocation with a maximal response at 200 μg/ml. Together, these results indicate that AOPPs induce ROS generation by increasing NADPH oxidase activity.

**Activation of IKKβ, IκBα and NF-κB**

The NF-κB pathway is a key component of the cellular response to a variety of extracellular stimuli. Therefore, we assessed whether AOPPs play a role in the regulation of this transcription factor by examining the phosphorylation of IKKβ, IκBα and NF-κB and the degradation of IκBα in MCs. The results showed a significant dose-dependent increase in IKKβ, IκBα and NF-κB phosphorylation and degradation of IκBα compared to the respective control group, without any change in the total protein content of IKKβ and NF-κB. Pretreatment of MCs with SOD (ROS inhibitor) almost completely inhibited NF-κB activation, so AOPP-induced ROS production and the main signaling pathway are involved in NF-κB activation (Fig. 3A, B and C).

**AOPPs induce MCP-1 mRNA and protein expression**

To determine the effect of AOPPs on MCP-1 expression, MCs were treated with AOPPs for up to 24 h, and then the MCP-1 activity and protein expression in the cell medium were determined by ELISA. Exposure to AOPPs induced MCP-1 protein expression in a dose-
dependent manner (Fig. 4A), and a significant increase in MCP-1 protein expression was observed after 24 h of incubation. This result suggests that AOPP-mediated induction of MCP-1 expression occurs at the transcriptional and translational levels. To further examine whether the increase in MCP-1 activity by AOPPs was a result of increased MCP-1 mRNA expression, MCP-1 mRNA expression in the MCs was determined by q-PCR. AOPPs induced MCP-1 mRNA accumulation in a dose-dependent manner in the MCs (Fig. 4B). These results further suggest that AOPPs induce MCP-1 expression at the transcriptional and translational levels.

Treatment with SLs Prevents AOPP-induced MCP-1 mRNA and protein expression

MCP-1 protein expression in the MCs was significantly up-regulated by AOPPs (200 μg/ml) compared to the control group. In addition, treatment with the NF-κB inhibitor SLs (5 and 10 μmol/L) reduced the AOPP-mediated increase in MCP-1 protein expression in a dose-dependent manner in the MCs (Fig. 4C), suggesting that NF-κB signaling participates in this regulation. Thus, SLs have clear effects on MCP-1 production in MCs, indicating that SLs are involved in reducing inflammation. To further examine whether the decrease in MCP-1 caused by SLs resulted from decreased mRNA expression, the MCP-1 mRNA expression in MCs was determined by qPCR. MCP-1 mRNA expression was significantly up-regulated after incubation with AOPPs (200 μg/ml) compared to the control group. However, treatment with the NF-κB inhibitor SL (5 and 10 μmol/L) blocked the AOPP-mediated increased in
MCP-1 mRNA expression. These results suggest that SLs reduce MCP-1 expression at the transcriptional and translational levels (Fig. 4D).

**Treatment with SLs prevents AOPP-induced IκBα degradation**

We further analyzed whether factors in the NF-κB activation cascade are influenced by SLs by assessing their effect on IκB proteins using Western blot analysis. MCs were preincubated for 1 h with 5 or 10 μmol/L of SLs and then subsequently incubated with 200 μg/ml AOPPs for 1 h. The results showed that preincubating the cells with 5 (Fig. 5A)
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Discussion

This study is the first to demonstrate that AOPPs induce NF-κB activation and MCP-1 expression at the gene and protein levels in MCs, thus confirming the participation of this transcription factor in the pathogenesis of DN. In agreement with previous reports indicating that NF-κB is required for the full induction of MCP-1 mRNA and protein expression [19], we showed that 200 μg/ml of AOPPs can stimulate a significant increase in MCP-1 mRNA and protein expression through the NF-κB pathway in MCs.

Fig. 5. SLs prevented AOPP-induced IκBα degradation. MCs were preincubated for 1 h with 5 or 10 μmol/L of SLs. Subsequently, the MCs were incubated with AOPPs (200 μg/ml) for 1 h. The AOPPs induced IκBα degradation in cultured MCs. The SLs prevented AOPP-induced IκBα degradation. The effect 10 μmol/L of SLs (B) was better than 5 μmol/L (A) for preventing AOPP-induced IκBα degradation. The data are expressed as the mean ± SD of three independent experiments. *p<0.05 vs. control, **p<0.05 vs. AOPP-treatment group.

or 10 μmol/L (Fig. 5B) of SLs prevented AOPP-mediated IκBα degradation. These results suggest that SLs can protect IκBα from proteolysis by the 26 S proteasome and therefore may interfere with a common step in the signaling cascade leading to NF-κB activation.
Fig. 6. AOPP-mediated signaling linked to MCP-1 expression in MCs. AOPP-induced MCP-1 expression is mediated through ROS leading to NF-κB activation. This signaling pathway might contribute to sustained MCP-1 expression, which has been implicated in DN inflammation. Moreover, SLs can reduce IκB degradation, preventing NF-κB activation and subsequent MCP-1 mRNA and protein expression.

Our results revealed that AOPP treatment of MCs stimulated cellular ROS production by activating NADPH oxidase. NADPH oxidase consists of four cytosolic regulatory subunits (p40phox, p47phox, p67phox, and the small GTPase Rac) and two membrane subunits (p22phox and gp91phox) [18]. Moreover, the specific up-regulation of p47phox, p22phox, and Nox4 has been demonstrated in the kidneys of diabetic patients [20]. This study found that NADPH oxidase activation was due to the translocation of p47phox from the cytosol to the cell membrane. In addition, AOPP treatment induced a significant increase in the ROS level. Previous studies have reported that ROS regulate NF-κB activation [21]. Therefore, we hypothesize that the AOPP-associated activation of the NF-κB signaling pathway occurs through the IKK pathway in MCs. In our study, AOPP-induced MCP-1 mRNA and protein expression and promoter activity were attenuated by SL pretreatment.

The NF-κB family comprises five members, including relA (p65), relB, c-Rel, p105/50, and p100/52, which associate as homo- or heterodimers and are regulated by diverse transduction cascades [22]. The subcellular location of NF-κB is controlled by a family of inhibitory proteins, IκB proteins (IκBs), such as IκBα and IκBβ, which bind to NF-κB and mask its nuclear localization signal, thereby preventing nuclear uptake. IκBs are phosphorylated by the IKK complex, which leads to ubiquitination and subsequent degradation by the 26S proteasome. Generally, proteins in the cytosol and nucleus are degraded via the ubiquitin-proteasome pathway, and the 26S proteasome is the central protease of the ubiquitin-dependent pathway of protein degradation and is formed by a barrel-shaped 20S core complex and two polar 19S complexes to catalyze protein degradation [23, 24]. Finally, the free NF-κB complexes translocate into the nucleus for target gene transcription activation, and the IκBs are degraded [25]. We demonstrated that AOPPs induce the phosphorylation of IKKβ and IκBα, resulting in IκBα degradation and sequela of NF-κB activation in MCs.

Inflammatory gene expression is regulated by several transcription factors, including NF-κB. Activation of this transcription factor is a very important step in the development of DN [26]; therefore, the pharmacological modulation of this process is the main objective in the design of new therapies for DN. In this work, we analyzed the effects of the natural compound parthenolide, which is an NF-κB inhibitor, and found that it reduces IκB degradation, preventing NF-κB activation and subsequent MCP-1 mRNA and protein expression. The inhibition of DNA binding occurs concomitantly with the inhibition of IκB degradation. This finding is in agreement with previous studies on the regulation of interleukin (IL)-8, ICAM-1 and VCAM-1 by parthenolide in several different cell types [27]. To the best of our knowledge, this study is the first to show that SLs protect against DN mainly through NF-κB inhibition (Fig. 6).

The synthesis of SLs was initiated with the easily available PTL (Fig. 1B). PTL was treated with p-toluenesulfonic acid (TsOH) to obtain MCL and was then subjected to epoxidation with m-CPBA to yield compound 1. This compound was then subjected to elimination with POCl3/
pyridine to form arglabin (Fig. 1C). PTL was treated under aqueous conditions to generate compound 2. Dehydrocostus lactone was treated with a standard Simmon-Smith reaction. The 11,13-conjugated double bond of the dehydrocostus lactone compound was protected as a Michael adduct [28], and the compound was then converted to a new compound using a Simmon-Smith reaction. Finally, the conjugated double bond was recovered by elimination to create the target compound (compound 3). Compounds 4 and 5 were obtained by the epoxidation of IAL and alantolactone, respectively, with m-CPBA (Fig. 1D) following the previously reported method [29].

Compared to PTL, the other natural and synthetic compounds were substantially more stable and had fewer side effects, although PTL and the other natural and synthetic compounds had similar bioactivities. Thus, the other natural and synthetic compounds may have a broad use in future clinical applications.

In summary, our results suggest that AOPPs induce MCP-1 mRNA and protein expression through a ROS/NF-κB-dependent pathway in MCs. Importantly, we found that SLs can exert a beneficial effect in AOPP-induced inflammation and ECM deposition by preventing NF-κB activation in MCs. The natural and synthetic compounds could provide a novel therapeutic approach for treating inflammation during DN.

**Conflict of Interest**

The authors declare no competing financial interests.

**Acknowledgments**

The present study was supported by the National Natural Science Foundation of China (NSFC) (NO. 81072848 to H.-B. L., NO. 21072106 to Y.C. and 81001377 to Q.Z.) and the Fok Ying Tong Education Foundation (No. 122037).

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